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Two forms of *Vibrio cholerae* O1 El Tor hemolysin derived from identical precursor protein

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Abstract

Vibrio cholerae O1 grown in heart infusion broth produces two forms of El Tor hemolysin (ETH) monomers of 65 and 50 kDa. These monomers form several different sizes of mixed oligomers ranging from 180 to 280 kDa in the liposomal membranes. We found that the N-terminal amino acid sequences, NH_2-Trp-Pro-Ala-Pro-Ala-Asn-Ser-Glu, of both the 65- and 50-kDa toxins were identical. We assumed, therefore, that the 65- and 50-kDa toxins were derivatives of the identical precursor protein and the 50-kDa protein was a truncated derivative of 65-kDa ETH. To substantiate this assumption, we treated the 260-kDa oligomer with trypsin and obtained a 190-kDa oligomer. This 190-kDa oligomer consisted of only the 50-kDa subunits. Both 260- and 190-kDa oligomers formed ion channels indistinguishable from each other in planar lipid bilayers. These results suggest that the essential part of the ETH in forming the membrane-damaging aggregate is a 50-kDa protein.

Keywords: El Tor hemolysin; Channel; Membrane; Pore-forming toxin; (*Vibrio cholerae* O1)

1. Introduction

Vibrio cholerae O1 produces several types of toxins, which are major virulence factors in cholera disease [1]. Among these, the primary virulence factor underlying the pathogenesis deriving from *V. cholerae* O1 is believed to be the cholera toxin. Secondary virulence factors are thought to be zonula occludens toxin [2], accessory cholera enterotoxin [3], and El Tor hemolysin (ETH, [4]). ETH appears to be unique among these toxins, as it causes hemolysis and cytolysis [4–10]. On the one hand, it was reported that ETH is less important in the pathogenesis of cholera disease, because the *V. cholerae* O1 mutant lacking both the cholera toxin and ETH induced mild diarrhea in healthy volunteers [11]. It is, however, unclear whether or not ETH causes diarrhea, because the experiment with volunteers was not performed using a mutant producing only ETH. *V. cholerae* non-O1 hemolysin, which is biologically, physicochemically, and immunologically indistinguishable from ETH [12,13], causes cytolysis in the cultured cell lines [14] and fluid accumulation in the rabbit ligated intestinal loops and intact intestines of suck-

Abbreviations: ETH, El Tor hemolysin; PC, phosphatidylcholine; TEN, 50 mM Tris-HCl–1 mM EDTA–3 mM sodium azide; HI, heart infusion; 2-ME, 2-mercaptoethanol

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ling mice [15]. Therefore, it is still possible that ETH could be a factor, which contributes to the pathogenesis of cholera.

The 79-kDa El Tor pro-hemolysin was secreted from *V. cholerae* O1 into the medium and subsequently is processed by protease, yielding mature 65-kDa ETH monomer [16,17]. The ETH monomer aggregates into the oligomer in the liposomal and rabbit erythrocyte membranes [5,6,9,10]. We found that *V. cholerae* O1 grown in heart infusion (HI) broth produces a large amount of 50-kDa hemolysin that is most likely the same species of ETH reported earlier [8]. However, it is not clear whether 65- and 50-kDa hemolysins are derived from the same precursor or are different proteins. We report here our finding that the 50-kDa protein is a truncated form of 65-kDa toxin.

2. Materials and methods

2.1. Purification of ETH

ETH was purified as described previously [5,18]. *V. cholerae* O1, biotype El Tor, serotype Inaba, strain N86 was grown in 150 ml of HI broth or syncase medium with 3% of glycerol in a 1000 ml Roux bottle, and incubated statically for 48 h at 30°C. Culture supernatant was mixed with ammonium sulfate to achieve 60% saturation and maintained for 18 h at 4°C. The centrifuged pellets were dissolved in 50 mM Tris-HCl, pH 8.0 and were applied to a Sephadex G-100 column (4.5×80 cm, Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM Tris-HCl–1 mM EDTA–3 mM sodium azide, pH 8.0 (TEN) and the column was eluted by the same buffer. Hemolytic activity of the eluate was measured as described previously [19]. Fractions containing the hemolysin were applied onto a Sephadex G-100 superfine column (2.5×85 cm, Pharmacia Biotech) equilibrated with TEN containing 250 mM glucose and the column was eluted by the same buffer. The purified hemolysin monomers appeared as a homogeneous band with an apparent molecular mass of 65 kDa as assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2. Planar lipid bilayer technique

Bilayer membranes were formed by the procedure reported by Benz et al. [20] on a Teflon film (25 μm in thickness) with a 0.2–0.3 mm orifice separating two 1.5-ml Teflon chambers. The orifice edge was precoated with 2 μl of PC-cholesterol mixture (2 mg lipid/ml, molar ratio of 1:1) in hexane. Planar lipid bilayers were formed by applying 5 μl of PC-cholesterol mixture (10 mg lipid/ml, molar ratio of 1:1) in *n*-decane by a brass wire sleeved with Teflon tubing onto an orifice of the septum immersed in the salt solution in the chambers.

2.3. Single-channel conductance measurements

Formation of lipid bilayer membranes was monitored from increase in the electrical capacitance to about 0.5 μF/cm^2. Electrical resistance of the membrane was consistently higher than 400 GΩ/cm^2. The chamber bathing a 10 GΩ probe was defined as the *cis*-compartment. ETH was added to the *trans*-compartment, stirred by a magnetic bar for 30 sec. The conductivity was recorded through calomel electrodes connected to a patch-clamp amplifier CEZ-2200 (Nihon-Kohden, Tokyo, Japan) by the voltage-clamping mode.

2.4. Preparation of liposomes

Liposomes were prepared as described previously [21]. In brief, 5 μmol of lipids (PC, 2.5 μmol and cholesterol, 2.5 μmol) were dried at the bottom of a tube, then suspended in 500 μl of the indicated solution. The suspension was subjected to sonic oscillation for 10 min at 20 W by a Branson Sonifier-200 equipped with a microtip.

2.5. Polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli [22] with a separating gel containing 6.5% of acrylamide.

2.6. Trypsin treatment of ETH oligomer

The 65-kDa ETH monomer (830 ng in 10 μl of TEN) mixed with PC-cholesterol liposomes (1×10^{-7} mol of lipid in 10 μl of 10 mM HEPES–150 mM NaCl, pH 7.2; lipid molar ratio of 1:1) was maintained for 2 h at 23°C. The mixture solubilized with 40 mM *n*-octyl-β-D-glucoside (Dojindo Laboratories, Kumamoto, Japan) was incubated with a 100-fold (w/w) higher concentration of trypsin (83 μg in 10 μl of 40 mM *n*-octyl-β-D-glucoside-10 mM HEPES–150 mM NaCl, pH 7.2; activity, 4840 USP units/mg) for 15 min at 23°C.

2.7. N-terminal amino acid sequence analysis

N-terminal amino acid sequence of the ETH proteins blotted on the polyvinylidene difluoride membrane was analyzed by Edman degradation using an automated protein sequencer (473A Protein Sequencer; Perkin-Elmer Applied Biosystems, Foster City, CA).

3. Results

3.1. Secretion of two active forms of hemolysin by V. cholerae O1

When *V. cholerae* O1 N86 was grown in Syncase medium, ETH with a molecular mass of 65 kDa was predominantly secreted (see Fig. 2 below, lane 2). A trace amount of 50-kDa toxin was also detected in the same preparation by immunoblotting [5]. In contrast, the same strain grown in HI broth consistently produced two forms of ETHs, with an apparent mass of 65 and 50 kDa (Fig. 1, lane 3). Polyclonal antibody raised against the 65-kDa ETH oligomer reacted with both the 65- and 50-kDa monomers [5]. N-terminal amino acid sequence of the 50-kDa protein was NH_2-Trp-Pro-Ala-Pro-Ala-Asn-Ser-Glu, which is identical to that of the 65-kDa ETH. Thus, we concluded that the 50-kDa protein is a derivative of the 65-kDa mature hemolysin cleaved off the 15-kDa C-terminal peptide. Attempts have been made to detect the 15-kDa fragment by 10–15% SDS-PAGE and immunoblotting without success. It is likely that the 15-kDa fragment was further degraded to smaller fragments by the proteases.

3.2. The heterosubunit oligomers formed by the 65- and 50-kDa monomers

To test whether the 65- and 50-kDa ETHs assemble co-aggregates in the membrane, the hemolysins purified from the culture supernatant of *V. cholerae* O1 N86 in HI broth were mixed with PC-cholesterol liposomes and the materials were subjected to SDS-PAGE analysis. We found several protein bands with a high molecular mass ranging from 180 to 280 kDa (Fig. 1, lane 4). Upon heating in the presence of SDS, these oligomers dissociated into two protein bands of 65 and 50 kDa (Fig. 1, lane 5), confirming the findings of a previous report [5]. ETH, purified from the culture supernatant in Syncase medium, with a molecular mass of 65 kDa predominantly formed a 260-kDa aggregate (see Fig. 2, lane 3). These results suggest that the 65- and 50-kDa monomers may form the co-aggregates.

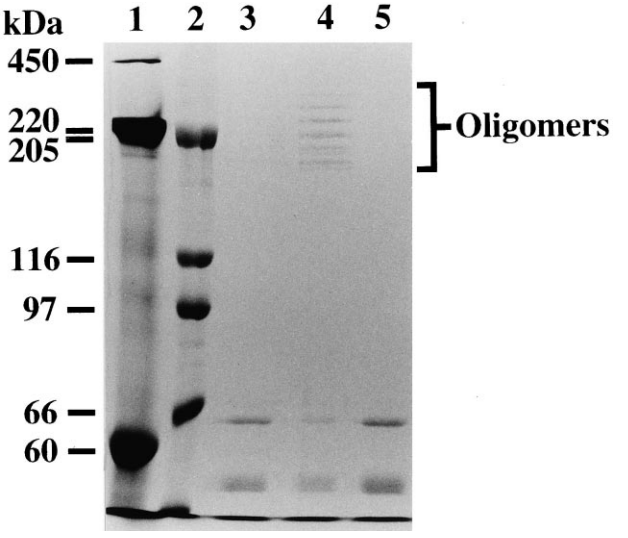

Fig. 1. Production of 50-kDa ETH protein by *V. cholerae* O1 grown in HI broth. Lanes: 1, Molecular mass markers without 2-mercaptoethanol (2-ME, see [5]); 2, molecular mass markers with 2-ME; 3, purified ETH monomer (3 μg in 20 μl of TEN); 4, ETH monomers (3 μg in 20 μl of TEN) were mixed with PC-cholesterol liposomes (2×10^{-7} mol of lipid in 20 μl of 10 mM HEPES–150 mM NaCl, pH 7.2, lipid molar ratio of 1:1), kept at 23°C for 2 h. The sample was unheated; 5, the same as in lane 4, heated at 100°C for 5 min.

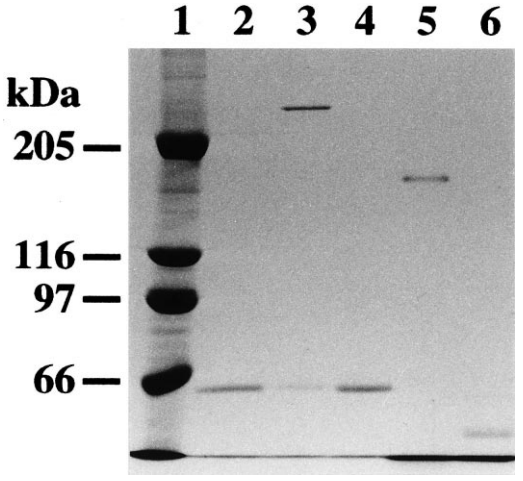

Fig. 2. Trypsin treatment of ETH oligomers. Lanes: 1, Molecular mass markers; 2, ETH monomers purified from syncase medium (830 ng in 10 μl of TEN); 3, ETH monomers were mixed with the liposome (830 ng ETH in 10 μl of liposome solution) and incubated at 23°C for 2 h; 4, the same as in lane 3, heated at 100°C for 5 min; 5, trypsin-treated ETH oligomers (830 ng ETH monomers in 20 μl of liposomes were treated with 10 μl of 83 μg trypsin in the presence of 40 mM *n*-octyl-β-D-glucoside); 6, the same as in lane 5, heated at 100°C for 5 min.

3.3. *In vitro demonstration of two forms of oligomers*

To explore the possibility that several sizes of oligomers can be derived from two forms of monomers, we conducted the following experiments. The toxin oligomers were formed by the monomers obtained from the culture supernatant of Syncase medium, dissolved in 40 mM *n*-octyl-β-D-glucoside, and were treated with trypsin. The trypsin-treated sample lost the native oligomer and yielded a new protein band at the 190-kDa position (Fig. 2, lane 5). This result indicates that the native oligomers were digested with trypsin yielding an oligomer of a different size. The trypsin-digested oligomers were dissociated into a homogeneous monomer with an apparent molecular mass of 50-kDa upon heating at 95°C in 1% of SDS (Fig. 2, lane 6). This 50-kDa protein band was indistinguishable from the 50-kDa toxin band obtained from the culture supernatant of HI broth (Fig. 1, lane 3).

To test a time-dependent conversion of 65- to 50-kDa molecule, the 65-kDa ETH was mixed with the culture supernatant of *V. cholerae* O1 N86 grown in HI broth. We demonstrated the time-dependent generation of the 50-kDa fragment derived from 65-kDa ETH monomer by immunoblotting (data not shown). The smaller, 15-kDa, fragment was undetectable even in 15% of acrylamide gel that is probably due to further degradation.

We reported earlier that the molecular mass of ETH oligomer was about 350 kDa [23]. We re-examined the molecular masses of oligomers by SDS-PAGE in various concentrations of acrylamide (Table 1). We obtained a new value of 260 kDa for the oligomer formed by 65-kDa ETH (Fig. 2, lane 3).

Table 1
Molecular masses of native and trypsinized oligomers in various concentrations of acrylamide gel

Acrylamide gel concentration	Apparent molecular mass (kDa)		
	Native oligomer[a]	Trypsinized oligomer	
(%)	Unheated	Unheated	Heated[b]
5	230	–	–
5.75	260	180	–
6.5	280	180	50
8	270	210	51
10	–	–	50
Average	260	190	50

[a]The oligomers were formed by 65-kDa ETH monomers in PC-cholesterol liposome membranes.
[b]Trypsinized oligomer was heated at 95°C for 2 min in SDS.

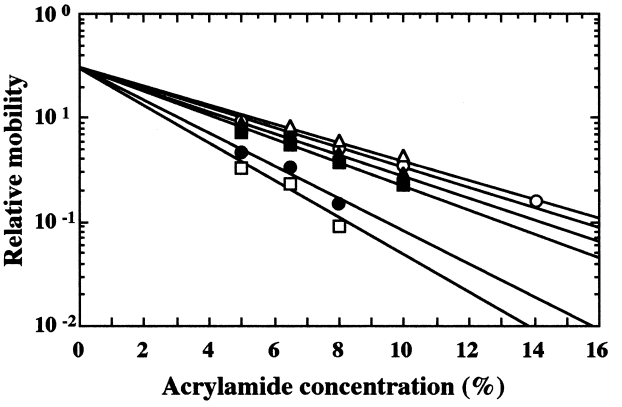

Fig. 3. Relative mobility of ETHs in various concentration of acrylamide. Electrophoresis was performed as described in the text in different concentration of acrylamide and the relative mobility was plotted against acrylamide concentration (Ferguson plot). ○, 65-kDa ETH monomer; Δ, the truncated 50-kDa ETH monomer; □, 260-kDa ETH oligomer; ●, 190-kDa ETH oligomer; ▲, 97.4-kDa phospholipase b subunit from rabbit muscle; ■, 66-kDa albumin from bovine serum.

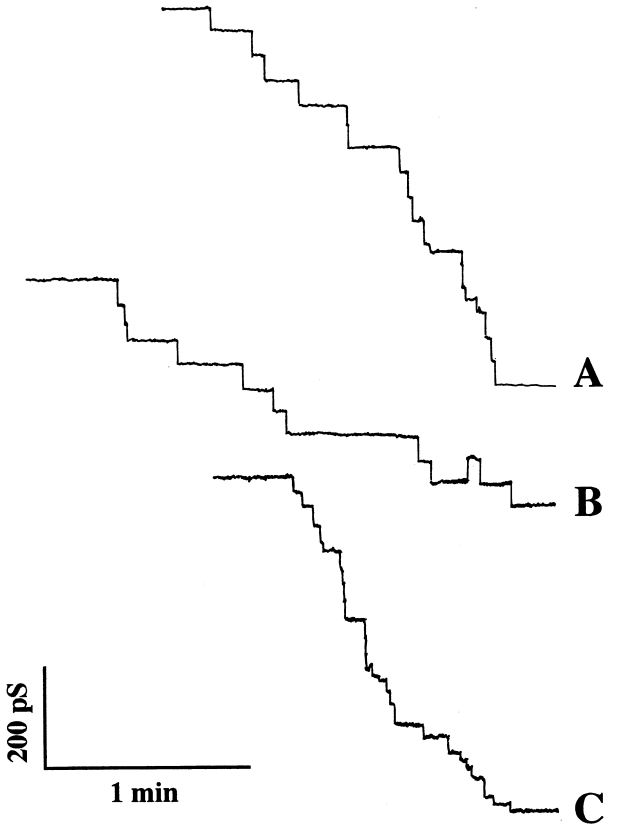

Fig. 4. Single-channel recordings of ETH oligomers in planar lipid membranes. Single-channel conductances of the oligomers were recorded with PC-cholesterol membranes bathed in 150 mM KCl–2.5 mM HEPES (pH 7.2). (A) 65-kDa ETH monomers (50 ng in 5 μl of TEN). (B) 65-kDa ETH monomers (830 ng in 10 μl of TEN) and PC-cholesterol liposomes (1×10^{-7} mol of lipid in 10 μl of 10 mM HEPES–150 mM NaCl, pH 7.2, lipid molar ratio of 1:1) were mixed and incubated for at 23°C for 2 h. The mixture solubilized in *n*-octyl-β-D-glucoside was added to the bath solution. (C) The same sample as in B was treated with trypsin at 23°C for 15 min in the presence of 40 mM *n*-octyl-β-D-glucoside. Membrane potential was +50 mV. Toxin was added to the *trans*-compartment of the Teflon cell.

Similarly, the molecular masses of the trypsin-digested oligomer and the heat-dissociated monomer were assessed to be 190 and 50 kDa, respectively. These results suggest that both oligomers are tetrameric aggregates. To ascertain if the electrophoresis and the molecular mass determination were done under optimum conditions, we plotted log relative mobility of ETHs versus acrylamide concentrations, the Ferguson plot [24]. Results showed in Fig. 3 that all the plots merged to one point at an acrylamide concentration of 0%, suggesting that the electrophoresis was carried out under controlled conditions and molecular masses of ETHs are reliable.

3.4. Channel activity of ETH oligomers incorporated into planar lipid membranes

For a better understanding of the cytolytic activity

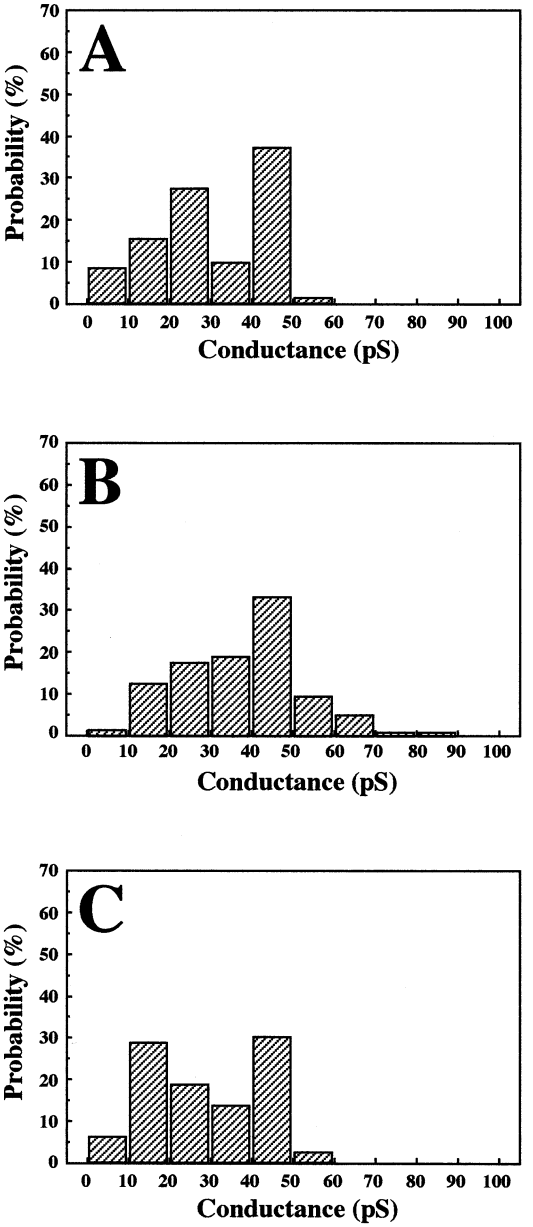

Fig. 5. Histogram of channel conductances of the oligomers formed in situ. (A) 65-kDa ETH monomers were added to the bath solution. (B) Data were collected from the same experiment as in Fig. 4B. (C) Data from Fig. 4C. Each experiment was recorded over 200 channel events.

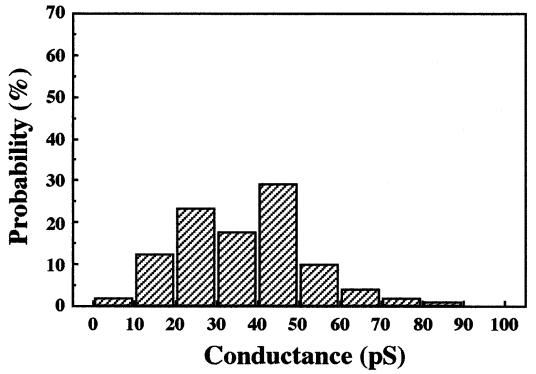

Fig. 6. Histogram of channel conductances of the oligomers formed by the mixture of 65- and 50-kDa ETH monomers. The mixture of the 65- and 50-kDa hemolysins (30 ng in 5 μl of TEN) was added into the bath solution containing 150 mM KCl–2.5 mM HEPES, pH 7.2. The experimental conditions were the same as in Fig. 4. Each experiment was recorded over 200 channel events.

of hemolysin, we tested the channel activity of the ETH oligomers formed under various conditions using single-channel conductance measurements. The 260-kDa oligomers assembled in the liposomal membrane were dissolved in 40 mM *n*-octyl-β-D-glucoside and were added to a chamber with a planar lipid bilayer membrane. We observed stepwise increments of membrane conductance as shown in Fig. 4B. When the single-channel measurement was carried out with a 190-kDa oligomer derived by the trypsin treatment of the 260-kDa protein, the channel conductances increased in a stepwise manner similar to those of the untreated 260-kDa oligomers (Fig. 4C). Single-channel conductances of the 260-kDa oligomers assembled in the bilayers in situ from the 65-kDa ETH monomers were indistinguishable from those obtained from the pre-assembled oligomers (Fig. 4A). A histogram of the single-channel conductances of the oligomers assembled in situ from the 65-kDa monomers and pre-assembled 260-kDa oligomers exhibited two peaks at 20 and 40 pS in 150 mM KCl–2.5 mM HEPES, pH 7.2 (Fig. 5A,B). The 190-kDa oligomers derived by the trypsin treatment of 260-kDa protein showed two conductance peaks at 10 and 40 pS in the same solution (Fig. 5C). It is, however, suggested that the small conductances of 10 and 20 pS, obtained from 190- and 260-kDa oligomers, were probably the same, because a similar experiment carried out in 1 M NaCl did not distinguish between them.

3.5. Channel activity of ETH oligomers formed in the presence of both 65- and 50-kDa ETH monomers

We investigated the channel activity of the oligomers, which were formed in the presence of both 65- and 50-kDa ETH monomers. Distribution of the single-channel conductances obtained from the mixture of the two proteins is shown in Fig. 6. The channel size and distribution were indistinguishable from those of the oligomers derived from the 65-kDa ETH alone (see Fig. 5A).

4. Discussion

The proteinacious cytocidal toxin, designated El Tor hemolysin, is encoded by the *hlyA* gene of the *V. cholerae* chromosome [12,13,17]. The toxin protein is secreted as 79-kDa pro-hemolysin into the medium and the proteolytic cleavage of the pro-hemolysin yields a mature 65-kDa hemolysin [16,17]. It has been reported that the water-soluble 65-kDa toxins form oligomeric aggregates of 250–350 kDa in the biological and artificial membranes [5,9,10,23]. We confirmed in this study that the apparent molecular mass of the oligomer is 260 kDa.

V. cholerae O1 El Tor N86 grown in HI broth produces two kinds of ETH of 65 and 50 kDa [5]. Using SDS-PAGE, we detected several sizes of oligomers formed by both ETH monomers. Trypsin treatment of the various sizes of oligomers generated a smaller oligomer of about 190 kDa, which dissociated into 50-kDa monomers upon heating in SDS. The amino acid sequence of the N-terminal end of the 50-kDa protein was identical to that of 65-kDa ETH. It is therefore suggested that the trypsin-cleavage site of the 65-kDa ETH may be located in the C-terminal domain. Thus, it appears that the essential domain for oligomer and channel formation is located in the 50-kDa protein. The conductance measurements in the planar lipid bilayer revealed that channel properties of 190- and 260-kDa oligomers were indistinguishable. Moreover, channel activities of ETH oligomers formed in the presence of 65- and 50-kDa ETH monomers were similar to those of 65-kDa ETH. It is, however, unclear whether these channel activities are derived from the 260-kDa oligomers or the co-oligomers of 50- and

65-kDa ETHs. We prefer to interpret this result to mean that several sizes of oligomers in the preparation form the channels with the identical conductances.

It has been reported recently that ETH monomers of 50–52 kDa produced by *V. cholerae* O1 form oligomeric aggregates of 150–250 kDa in erythrocyte membranes [6,8]. This means that the 15-kDa C-terminal portion of the 65-kDa ETH monomer does not play an essential role in the formation of ion permeable channels. In addition, the 150–250-kDa aggregate forms a transmembrane pore with a diameter of about 1.4 nm [8], which is consistent with the previous report that the oligomer formed by 65-kDa ETH monomers was 1.2–1.6 nm [5]. These results were in accord with the fact that 190-kDa oligomers form large ion conductive channels in planar lipid bilayers.

One may ask whether or not the 50-kDa ETH contributes to the pathogenesis of *V. cholerae* O1. However, no one can clearly answer this question, because isolating only the 50-kDa toxin from the culture supernatants has not been achieved yet. It was reported that *V. cholerae* non-O1 N037 produces two forms of mature non-O1 and 48-kDa hemolysins [25]. The latter is most likely a truncated form of the mature toxin. Hemolytic activity of the 48-kDa hemolysin was low as compared with that of mature hemolysin [25]. It is, therefore, likely that the 50-kDa ETH is comparable fragment to the 48-kDa non-O1 hemolysin. It is difficult, if not impossible, to test the role of 50-kDa toxin only to the pathogenesis of *V. cholerae* O1, since pathogenesis might be the sum of many virulent factors. However, it must be emphasized that our 50-kDa protein can damage the membrane and form the aqueous pore in the planar lipid membrane.

A recent study reported that mannose-sensitive hemaggutinin is involved in generation of the 65-kDa ETH from 79-kDa pro-ETH [16]. If the 65-kDa ETH was further processed by the monnose-sensitive hemagglutinin, the 50-kDa ETH should be generated by not only *V. cholerae* grown in HI broth but also in Syncase medium. However, 50-kDa ETH species was not produced in the Syncase medium. This result suggests that mannose-sensitive hemagglutinin do not cleave off the 15-kDa C-terminal region of 65-kDa ETH monomer. It is likely, therefore, that another protease produced by *V. cholerae* is involved to cleave between 15- and 50-kDa ETH peptides.

Formation of the multiple oligomeric aggregates by the pore-forming proteins has been reported in complement C9 [26], staphylococcal alpha-toxin [27,28], and *Serratia marcescens* hemolysin [29]. In all of these cases, the multiple forms of the aggregates were formed by a single species of toxin, suggesting that these oligomers contain different numbers of identical subunits.

It is of interest to know how the water-soluble monomeric toxin forms the membrane-spanning oligomers and transmembrane pore. Since it was firmly established that bacterial porin forms water-filled large pores across the membranes by the β-barrel structure, we conducted a computer-aided analysis of the β-structure of ETH monomers by the AMPHI [30] and FALT [31] programs. It was shown by the programs consistently that an ETH monomer contains 15–18 segments of β-structure, suggesting a possibility that the protein can take a conformation to form transmembrane segments. We found, on the other hand, that ETH monomer contains many large hydrophilic segments, suggesting another possibility that the protein can also take the water-soluble configuration. Thus, the computer-aided secondary structural analysis supports the fact that ETH can take two alternative conformations.

In summary, it was concluded that both 65- and 50-kDa ETH monomers secreted from *V. cholerae* O1 form the tetrameric aggregates in the membrane, of which the apparent sizes were 260 and 190 kDa, respectively. Since the 260-kDa oligomer could be converted to 190 kDa upon trypsin treatment and N-terminal amino acid sequences were identical, we concluded that both proteins are derived from an identical precursor protein.

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